# Development and validation of a highly sensitive LC-MS/MS method for quantitation and confirmation of oxytocin in milk

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## ABSTRACT

Oxytocin activity in mammals include both uterogenic and galactogenic effects that result in uterine contractions and milk letdown respectively. The presented method supersedes the many methods for exogenous oxytocin analysis by enzyme immune assay (EIA), HPLC and LC- MS in terms of sensitivity and specificity. The workflow MRM triggered collection of EPI (Enhanced Product Ion) gives the same results and quantitative capabilities of routine MRM. Advantage of using the EPI data – results may be compared to the library for a fit score for an extra criterion of confirmation and can also be used to identify mimics of synthetic oxytocin.

## INTRODUCTION

India is the world's largest producer of dairy products by volume and has the world's largest dairy herd. The country accounts for more than thirteen percentage of world's total milk production and consuming almost all of its own milk production. Oxytocin (OT) is a nine amino acid peptide hormone synthesized in the hypothalamic neurons and released from the posterior pituitary gland. This hormone activity in mammals include both uterogenic and galactogenic effects that result in uterine contractions and milk letdown respectively. The injectable form of Oxytocin is FDA approved for use in veterinary medicine in multiple animal species. In India, Oxytocin is categorized as schedule H-drug under Drugs and Cosmetics rules, 1945. Hence it cannot be bought or sold without a prescription and its use by dairies made illegal. Also, it is specifically banned under the Prevention of Cruelty to Animals Act, 1960 section 12 of and the Foods and Drug Adulteration Prevention Act, 1960.

In the present study Quadrupole- Trap hybrid instrument was employed for quantification as well as confirmation of oxytocin present in milk sample. Solid phase extraction protocol was employed for sample extraction. Single injection workflow was used for quantification and confirmation of Oxytocin in milk samples. Enhanced product ion spectra generated by the instrument along with the MRM data is used as an additional confirmation tool to identify detected compounds by automated searching against mass spectral library. Library matching results are evaluated on the basis of purity, fit and reverse fit of the obtained MS/MS in comparison to the library spectrum.

## MATERIALS AND METHODS

**Sample Preparation:**

20 Milk samples were procured locally from Delhi, Punjab and Utter Pradesh, India and were analyzed for Oxytocin. Milk samples were mixed with 500 µl of 100 % TCA and centrifuged at 4000 RPM for 10 minutes. The precipitate was re-dissolved in 1ml of 0.25 % acetic acid and stirred for 15 minutes. 100 µl of 100% TCA is added and centrifuged. Supernatants were cleaned up using Phenomenex Strata C-18E cartridges and wash with Methanol: Water (30:70) followed by elution with Ethanol: 6N HCL (1000:1). Samples were dried under stream of nitrogen and dried residue was reconstituted in 1ml of methanol: Water (90:10)

**HPLC Conditions:**

LC separation was achieved using the Shimadzu prominence system with a Zorbax SB C-18 (4.6 × 1.5mm) 1.8 µm column with a gradient of water and methanol containing 0.1% formic acid at flow rate of 1.1 mL/min. The injection volume was set to 20 µL.

**MS/MS Conditions:**

The AB SCIEX QTRAP® 4500 was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in positive polarity. Two selective MRM transitions (1007.3 / 723; 1007.3 / 706.3) were monitored for oxytocin using the ratio of quantifier and qualifier ion for compound identification. Analyst® 1.6.1 software was used for method development and data acquisition. LC-MS/MS data was processed using the MultiQuant™ software version 2.1

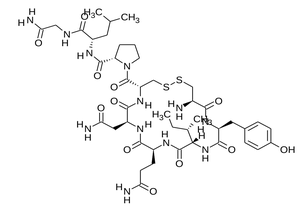

**Figure 1.** Structure of Oxytocin

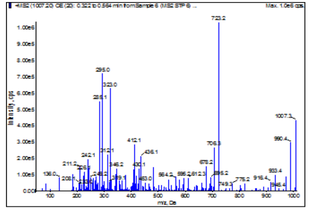

**Figure 2.** fragmentation pattern of Oxytocin

## RESULTS

Aqueous linearity with a linear dynamic range of 3.5 orders was made from set of standard dilutions from 0.05 ng/ml to 250 ng/ml injected in triplicates. Calibration curve was found linear in the above range with regression co-efficient (r): 0.9981 using linear regression and weighing factor $1/X^2$. Standard concentration, 0.005 ng/ml is set as the limit of detection with a signal to noise ratio of more than 10. Similarly matrix matched calibration curves were made with standard levels ranging from 0.05 ng/ml to 250 ng/ml with more than 3 order of linear dynamic range. Linear graph was obtained with regression co-efficient (r): 0.9984 by using weighing factor 1/X.

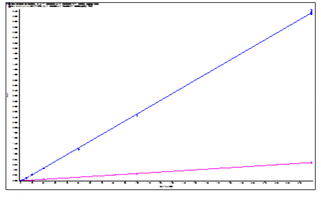

**Figure 3:** Linear range of the detection of Oxytocin from 0.05 to 250 ng/mL (r = 0.999)

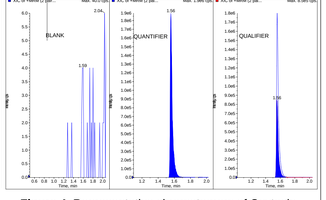


**Figure 4.** Representative chromatogram of Oxytocin.

| Standard ID | Area | Calculated Conc. | Accuracy (%) |
|---|---|---|---|
| Oxytocin 0.1 ppb | 422.6 | 0.108 | 108 |
| Oxytocin 0.1 ppb | 381.1 | 0.0904 | 90.4 |
| Oxytocin 0.5 ppb | 1338.4 | 0.496 | 99.2 |
| Oxytocin 0.5 ppb | 1477.1 | 0.555 | 111 |
| Oxytocin 1.0 ppb | 2401.4 | 0.946 | 94.6 |
| Oxytocin 1.0 ppb | 2598.9 | 1.03 | 103 |
| Oxytocin 10 ppb | 24412 | 10.3 | 103 |
| Oxytocin 10 ppb | 21166.8 | 8.89 | 88.9 |
| Oxytocin 50 ppb | 110897.6 | 46.9 | 93.8 |
| Oxytocin 50 ppb | 109552.6 | 46.3 | 92.6 |
| Oxytocin 100 ppb | 259294.8 | 110 | 110 |
| Oxytocin 100 ppb | 256709.2 | 109 | 109 |
| Oxytocin 250 ppb | 574694.4 | 243 | 97.3 |
| Oxytocin 250 ppb | 592463.3 | 251 | 100 |

**Table 1.** Accuracy of spiked standard dilutions.

| Spike Levels | Relative Standard Deviation (% CV) |
|---|---|
| At LOQ:0.05 ppb | 4.70% |
| At 2 X LOQ: 0.1 ppb | 3.50% |
| At 0.1 ppb (Repeated) | 5.00% |
| At 5 X LOQ: 0.5 ppb | 5.30% |
| At 10 X LOQ: 1ppb | 3.30% |

**Table 2.** Reproducibility data at LOQ, 2 X LOQ, 10 X LOQ & 20 X LOQ for 10 replicate injections

Method validation experimental set were processed as per EU SANCO/12495 directive recommendations. Results of accuracy data obtained for Oxytocin in the milk matrix is given in Table 1. Oxytocin eluted at RT of 1.46 minutes with minimum background noise in 5 minutes chromatographic run. Repeatability at LOQ level was evaluated by 10 repeated injections of 0.05 ng/ml. Repeatable injections (n=10) at LOQ gives the % relative standard deviation of 4.7%.

| QC sample ID | Area | Recovered Conc. | Recovery (%) |
|---|---|---|---|
| 0.1 ppb | 440.1 | 0.115 ppb | 115 |
| 0.1 ppb | 431.7 | 0.112 ppb | 112 |
| 0.1 ppb | 418.4 | 0.106 ppb | 106 |
| 0.5 ppb | 1329.7 | 0.492 ppb | 98.4 |
| 0.5 ppb | 1313.9 | 0.485 ppb | 97.1 |
| 0.5 ppb | 1437 | 0.538 ppb | 108 |
| 1 ppb | 2438.7 | 0.962 ppb | 96.2 |
| 1 ppb | 2291.8 | 0.9 ppb | 90 |
| 1 ppb | 2405.9 | 0.948 ppb | 94.8 |

**Table 3:** Recovery data at 0.1 ppb, 0.5 ppb and 1.0 ppb spiked levels

| Parameter | What/ How | Criteria |
|---|---|---|
| Linearity | Through calibration graph | Accuracy within ±20% |
| Matrix effect | Comparsion of response between Aqueous and matrix samples | - |
| LOQ | Lowest level for which it has been demonstrated that criteria for trueness and precision have been met | ≤MRL |
| Specificity | Response in reagent blank and control samples | < 30% of LOQ |
| Robustness | Can be derived from ongoing method validation / verification through establishing average recovery and RSD | See above |
| Trueness (Bias) | Determine the average recovery for spike levels | 70-120% |
| Precision (RSD) | Determine within laboratory reproducibility | ≤ 20% |

**Table 4:** Method validation parameters and criteria as per SANCO

The inter- and intra-day precision of quality control (QC) samples at different spiking levels were checked and results were given in Table 2. Recovery of the extraction method is evaluated by analysing the milk samples spiked at different concentration levels. Calculated the amount of oxytocin recovered from the spiked milk samples using matrix matched calibration curve.

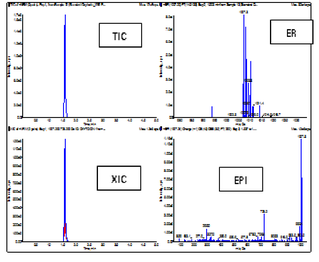


**Figure 5:** IDA Viewer shows both MRM and EPI data from sample generated by MRM to EPI information dependent acquisition work flow

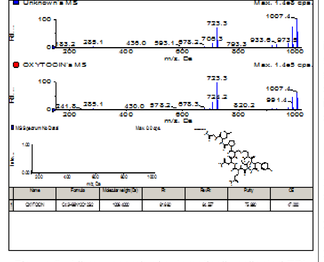


**Figure 6:** Library search of automatically collected EPI spectra of the Oxytocin with 76 % purity for QC spiked samples

Out of 20 milk samples analyzed, 9 showed traces of Oxytocin between concentrations 0.1 to 0.5 ng/ml.
Advantage of using the EPI data – results may be compared to the library for a fit score for an extra criterion of confirmation and can also be used to identify mimics of synthetic oxytocin.
Example: Spiking at 0.1 ng/ml; From 2 pg/ul Oxytocin standard, 50 ul was taken and spiked to 5 ml of milk sample.
Absolute amount spiked: 100 pg; Final reconstitution volume: 1ml
Final concentration of oxytocin in the reconstituted volume: 100 pg/ml or 0.1 ng/ml
Concentration obtained for QC sample spiked at LOQ level: 0.106 ng/ml
Therefore, Recovery % = (Concentration recovered from calibration table/Concentration spiked) X 100
= [(0.106 ng/ml)/ (0.1 ng/ml)] X 100 = 106 %

Post column infusion study was conducted in order to evaluate the suppression/ enhancement in the signal of Oxytocin in presence of matrix interferences. The result of this study proved that there is no enhancement or suppression of signal of Oxytocin at the retention time of the analyte in the developed method as shown in figure 8. Report showing calibration table including MRM ratio, calibration graph and peak review were generated with the help of Analyst companion software, Analyst reporter/ MultiQuant ™.

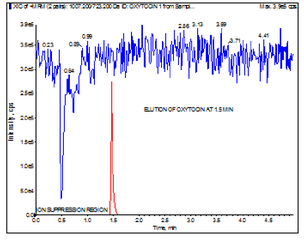


**Figure 7:** Matrix suppression/ enhancement study by post column infusion experiment

**Figure 8:** Example reports generated by MultiQuant™ Software

## CONCLUSIONS

The method and data presented here showcase the fast and accurate solution for the quantitation and identification of oxytocin in milk samples by LC-MS/MS. The AB SCIEX QTRAP® 4500 systems provide excellent sensitivity and selectivity for this analysis, with minimal sample preparation allowing maximized throughput for the analysis of many samples in a short time period. All the collected milk samples were screened and quantified for oxytocin. Automatic MRM ratio calculation in MultiQuant™ software was used for compound identification. Matrix interferences study was conducted to understand the matrix effects and EPI based search was used for high confidence result reporting in challenging matrices, such as milk.

## REFERENCES

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## TRADEMARKS/LICENSING